

The University of Chicago

AN ANTIGENIC POLYSACCHARIDE
FRACTION OF ASCARIS LUMBRICOIDES
(FROM HOG)

A DISSERTATION SUBMITTED TO THE
FACULTY OF THE DIVISION OF THE
BIOLOGICAL SCIENCES IN CANDIDACY FOR
THE DEGREE OF DOCTOR OF PHILOSOPHY

DEPARTMENT OF HYGIENE AND BACTERIOLOGY

By

DAN HAMPTON CAMPBELL

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INTRODUCTION

One of the most striking developments within recent years in immunology and bacteriology has been the immunochemical studies of specific polysaccharides. The results of these studies have brought out the importance of nonprotein substances in immunological reactions and have profoundly influenced conceptions of specificity. These investigations, however, have been concerned chiefly with bacteria and related organisms. Little if any consideration has been given to the significance of polysaccharides of parasitic or free-living animals with the possible exception of a recent report¹ by the present author describing the protective action of a variety of chemical fractions, including a polysaccharide, from *T. taeniaeformis* (cat tapeworm) and its larval form. The importance of carbohydrates in immunological reactions has become so widely recognized and so often summarized in various papers and textbooks that it seems unnecessary to review the field at this time. Nevertheless, it admittedly furnishes a basis for the present investigation.

Ascaris lumbricoides (of hog) was chosen for the present investigation because it is a form with which considerable work has been done regarding specificity and immunology. For example, Schwartz,² Hektoen³ and Canning,⁴ using saline extracts or "protein" fractions, found that many of the ascarids gave cross precipitin reactions. It is of interest to note that in no case has an immunological difference been found between *Ascaris lumbricoides* from man and hog although it is believed that the two are biologically distinct. A summary of the more important literature on the immunology of the ascarids has been given by Taliaferro.⁵ Besides the possible correlation with previous investigations, *Ascaris lumbricoides* is a form readily available in amounts suitable for large-scale chemical analysis.

Chemical study indicates that worm tissue contains at least two types of colloidal carbohydrates. One, which is not considered in this paper, is associated with the alcohol-soluble lipoids from which it can be isolated by saponification. The other, with which the present series of experiments is con-

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1. Campbell, D. H.: *Am. J. Hyg.* **23**: 104, 1936.

2. *J. Parasitol.* **6**: 115, 1920.

3. *J. Infect. Dis.* **39**: 342, 1926.

4. *Am. J. Hyg.* **9**: 207, 1929.

5. *The Immunology of Parasitic Infections*, New York, Century, 1929.

cerned, can be extracted from fresh worm material by aqueous solutions. This carbohydrate fraction, after rigorous purification, not only reacts with specific antibodies, but also incites their formation. The present paper deals chiefly with the problem of whether the polysaccharide is itself antigenic or whether the activity is due to otherwise undetectable traces of protein. Studies on the specificity are still in progress and will be reported in the near future.

EXPERIMENTAL

Chemistry

Isolation of the crude polysaccharide fraction.—The polysaccharide was isolated from worm material prepared in the following manner. Fresh worms were washed with tap water, frozen solid in the freezing chamber of the refrigerator and then rapidly passed through a fine "food grinder." Freezing and grinding were repeated until there was formed a fine tissue pulp which was stored in the freezing chamber of the refrigerator. (Although kept in a frozen condition the amount of polysaccharide gradually decreased.)

In general, two methods were used for the isolation of polysaccharide. The first consisted of suspending about 300 grams of the tissue pulp in one volume of distilled water to which was added 1 cc. of 40% NaOH and 5–10 cc. of ether. The p_H was approximately 8. This mixture was stirred vigorously for several hours at room temperature and then centrifuged at high speed for 45 minutes. The insoluble material was washed with a small volume of very dilute alkaline solution. The washing was added to the original extract and the residue discarded. Ten per cent trichloroacetic acid was then very cautiously added until a distinct cloudiness persisted. This was followed by the addition of 95% ethyl alcohol until a small but definite precipitate formed. Approximately 0.3 volume of alcohol was required. (The exact amount was dependent upon the p_H of the solution.) When the concentrations of acid and alcohol were properly adjusted, a voluminous precipitate, consisting mostly of protein, settled out after 12 hours in the refrigerator. This precipitate was centrifuged off and 1 to 1.2 volumes of 95% alcohol added to the solution. The crude polysaccharide fraction, which immediately precipitated, was then redissolved in a small volume of distilled water and centrifuged, and any insoluble material discarded. A trace of NaCl was then added to the solution and the polysaccharide again precipitated with 1 volume of 95% alcohol. Careful resuspension and precipitation were continued until the resulting product was perfectly soluble in distilled water. Such a procedure yielded a very soluble substance which was usually biuret-negative.

A second method,⁶ which consisted of digesting protein was carried out by suspending 200 to 300 grams of the tissue pulp in an equal volume of

6. This process yielded about twice the amount of polysaccharide, but had two disadvantages. Exposure to increased hydrogen ion concentration reduced the capacity to incite the formation of antibodies (*vide* anaphylaxis experiments) and the pepsin had to be free of polysaccharide. The pepsin used in the present investigation was a special product of the Wilson Laboratories, Chicago, and supplied through the courtesy of Dr. D. Klein. It had a U.S.P. value of 1:30,000.

0.1% pepsin solution. The p_H was adjusted to approximately 4.5 with HCl; toluol was added and the mixture allowed to stand at 37 C. until only a very small amount of insoluble material remained. With continual mechanical stirring, digestion was practically complete in 36 hours. The insoluble residue was then discarded and 2 to 3 volumes of 95% alcohol added to the solution. The resulting precipitate, which contained the crude polysaccharide, was partially purified by repeated precipitation with alcohol as previously described.

Purification.—The crude polysaccharide fraction, prepared as described above, usually contained 1.0 to 0.1% nitrogen. Preliminary study, however, showed that the last trace of detectable nitrogen could be removed by several methods. For example, precipitation of the polysaccharide with barium or calcium hydroxides, fractional precipitation with 95% alcohol or precipitation of the nitrogenous material with phosphotungstic acid. The latter method was chosen since it seemed the most efficient. It was carried out as follows. Four to five grams of the crude polysaccharide fraction were dissolved in 25 cc. of distilled water and placed in an ice bath. A solution containing 10% phosphotungstic acid and 1% sulfuric acid was then slowly added until no further precipitation occurred. The precipitate was removed by rapid suction filtration and discarded and the filtrate added to one volume of chilled 95% alcohol. The precipitate (polysaccharide) was immediately centrifuged off, resuspended in distilled water and any insoluble material carefully removed. A second treatment with the phosphotungstic acid mixture was always carried out although all nitrogenous material was usually removed with the first precipitate. Precipitation with alcohol was repeated as long as any water-insoluble material was obtained, and the final product dried with absolute alcohol and ether.

The first method of extraction yielded approximately one gram of purified carbohydrate from 100 grams of fresh tissue. The second method (peptic digestion) yielded about twice this amount.⁷

Chemical properties.—The purified product was pure white, non-hygroscopic, amorphous and contained a trace of ash which gave a positive test for phosphorous. No nitrogen could be detected by macro-Kjeldahl analyses of one to ten gram samples. The material readily dissolved in hot or cold water and formed neutral, opalescent solutions in concentrations of 1%. (For some unknown reason the degree of opalescence varied to a slight extent with different preparations.) Such solutions gave strong red-brown iodine reactions but no positive color tests for proteins. The substance was precipitated by 0.8 to 0.9 volumes of 95% ethyl alcohol and formed insoluble compounds with barium and calcium hydroxide. It was incompletely precipitated by saturation with ammonium sulfate. It was not precipitated by mineral acids, sodium hydroxide or basic lead acetate. There was no discoloration of the solution upon adding sodium hydroxide and heating. It

7. The amount of polysaccharide in pig ascaris was actually much greater than this. For example, boiling fresh worm material for two hours in 10% NaOH and precipitating the proteins with acid, yielded three to four grams of polysaccharide per hundred grams of wet tissue. This product, however, was immunologically inactive.

produced a high dextrorotation of polarized light and gave no evidence of reducing power as tested by heating with sodium picrate or Fehling's solution.

Further analysis was carried out as follows. A sample of the polysaccharide was hydrolyzed by boiling in 0.5 *N*. H₂SO₄ and the solution quantitatively neutralized with Ba(OH)₂. A maximum reducing power, as determined by Shaffer-Hartman, was reached after four to five hours and amounted to approximately 94%, calculated as glucose. This value altered only slightly in the next two hours of heating. The specific rotation was:

$$[\alpha]_{D^{24}} = \frac{1.15 \times 100}{1 \times 2.0} = +57.5^{\circ}$$

The hydrolysate gave none of the common reactions for hexuronic acids, pentoses or ketoses.

Freshly distilled phenylhydrazine was added to the hydrolysate and the mixture allowed to stand overnight in the refrigerator. There was no evidence of the formation of mannosehydrazone. After heating for 30 minutes in a boiling water bath and cooling, a voluminous precipitate of yellow crystals settled out. The crystalline structure was similar to that of phenyl-glucosazone. After two recrystallizations from 60% alcohol the osazone showed a melting (decomposition) point of 202–204 C. and a mixed melting point with pure glucosazone of 205 C. After removal of the osazone, the hydrolysate was treated with benzaldehyde, and after 12 hours the precipitate of benzaldehyde-phenylhydrazone was removed. The solution was then shaken with ether to remove the benzaldehyde and evaporated to dryness. The trace of solid material left after evaporation was not combustible.

The foregoing rough analysis of the polysaccharide indicates that it is composed largely of hexoses, presumably glucose, and that it is at least relatively free of hexuronic acids, pentoses, ketoses and mannose.

IMMUNOLOGY

Precipitin Tests

As the investigation progressed it became evident that the polysaccharide not only reacted with specific antibodies, but also incited their formation. The question immediately arose as to whether or not such activity was due to otherwise undetectable traces of protein. If protein were responsible for the activity, there should be cross reactions between the polysaccharide and the water-soluble protein fraction since the two were closely associated in the original aqueous extract. The problem was studied, using a protein fraction prepared in the following manner:

An aqueous extract of about 200 grams of the tissue pulp, prepared as described previously for the isolation of polysaccharide, was half saturated with ammonium sulfate. Trichloroacetic acid was then added until no further precipitation occurred, and the mixture allowed to stand over night in the refrigerator. The precipitate, which contained most of the extracted protein material, was resuspended in 0.9% NaCl and centrifuged. The material which failed to redissolve was discarded and the solution shaken with several changes of ether. The ether was then removed, the solution half saturated with ammonium sulfate and acidified with trichloroacetic acid to the point of maximum precipitation. After standing overnight in the

refrigerator, the mixture was centrifuged and the precipitate resuspended in dilute saline and again centrifuged. The trace of insoluble material was discarded, toluol added to the supernatant and dialysed relatively free of electrolytes (three days in distilled water). The solution, plus a fine precipitate, was then removed and evaporated to dryness in a current of warm air.

The resulting product was very soluble in dilute saline and contained approximately 15% nitrogen and 1% polysaccharide.⁸ Intravenous injection of over 0.0001 gram produced severe systemic reactions in 2 kilogram rabbits, but similar injections of less than 0.01 gram produced no visible reactions in guinea pigs weighing 300 grams.

Precipitin tests were then made of the nine possible combinations of the three ascaris antigens (whole worm, protein and polysaccharide) and their antisera. The antisera and tests were prepared as follows:

Sensitization of rabbits: whole worm material.—Each animal received three series of intraperitoneal injections of whole worm powder suspended in 0.9% NaCl. Each series consisted of five injections; the first, 0.1 gram of powder; the second, 0.2 gram; and the third, fourth and fifth, 0.3 gram, making a total of 3.6 gram per rabbit. A two day interval was allowed between each injection of a series and an eight day interval between each series.

Polysaccharide.—Each animal only received two series of intraperitoneal injections of polysaccharide. A total of approximately 0.4 gram was given. The time intervals were the same as previously described. The antigen was introduced intraperitoneally because, for some unknown reason, when introduced intravenously, the polysaccharide induced either a low precipitin titer or none at all in the resulting serum. The sera of three out of four rabbits showed similar titers (as listed in table 1) while the fourth, strangely enough, contained no precipitins.

Protein.—Rabbits were given two series of intraperitoneal injections as described above. The first injection consisted of 0.0001 gram of protein. The amount was gradually increased to 0.05 gram because with each succeeding injection the animals became less susceptible to the toxicity of the protein. A total of approximately 0.2 gram was given each rabbit.

Preparation of sera and test antigens.—The rabbits were not given fresh vegetables during the process of immunization or starved for more than ten hours before collecting blood.⁹ They were bled from the heart ten days after the last injection, the blood permitted to clot at room temperature and then stored in the refrigerator overnight. On the following morning the serum was removed, centrifuged and replaced in the refrigerator until used. Aseptic technic was used in handling the sera so that no preservatives were necessary.

The test solution for whole worm material was prepared by stirring a sample of the tissue pulp (previously described) with an equal volume of

8. No method was found whereby the last trace of polysaccharide could be removed without denaturing the protein. It was thought advisable, therefore, to use a preparation containing some polysaccharide rather than risk alteration of the protein.

9. In some instances when animals were given a diet of fresh vegetables the serum reacted with many types of colloidal carbohydrates to give precipitates. This was also noted in some animals which had been starved for 18 hours before bleeding. The nonspecific reaction was apparently characteristic for polysaccharides since foreign serum or ovalbumin produced no precipitation. Guinea pigs showed no passive sensitization when treated with such sera and subsequently tested with nonspecific polysaccharides.

0.9% NaCl. Such a saline extract contained a finely divided suspension which could not be removed by centrifugation or ordinary filtration. It was, however, possible to cause flocculation of this undesirable material by incubation at 45 to 50 C. for 45 minutes. After such treatment the suspension could easily be removed, leaving a clear satisfactory test solution. The protein and polysaccharide needed no preparatory treatment and were merely dissolved in 0.9% NaCl. Dilutions of antigens, as given in table 1, were calculated upon the basis of solids per unit volume of solution.

TABLE 1.—*Homologous and Heterologous Precipitin Reactions Between Three Types of Ascaris Lumbricoides (of Hog) Antigens (Whole Worm, Protein and Polysaccharide) and Their Antiserums*

Test Antigen	Antiserums		
	Whole Worm	Polysaccharide	Protein
Polysaccharide	12,800*	6,400	0
Protein	6,400	0	25,600
Saline whole worm extract	14,000	8,000	16,000

* The figures represent the average highest dilution of test antigen showing a reaction after 12 hours at 4 C. No cross reactions were observed between *M. expansa* (sheep tapeworm) and *Ascaris lumbricoides*.

Technic of precipitin tests.—The “ring” method was used. Approximately 0.1 cc. of diluted serum (1:2 with saline) was placed in 4×50 mm. tubes and carefully overlaid with an equal volume of the antigen solution. The tubes were incubated for 45 minutes in a 40 C. water bath and then allowed to stand for 12 hours in the refrigerator. At the end of this time the highest dilution of antigen which gave a distinct precipitate at the serum-antigen interphase was considered the titer.

The lowest dilution of antigen tested was 1.0%, i.e., 1:100. Starting with this concentration, dilutions were made in geometric progression, i.e., 1:100, 200, 400, etc.

Results of the precipitin tests.—It will be seen from the data listed in table 1 that the polysaccharide and protein fractions reacted only with homologous or whole worm antiserums. The two fractions were therefore apparently immunologically distinct. Cross reactions between polysaccharide and whole worm material and their antiserums demonstrated that the active principle of the former was also present in whole worm material and therefore not some substance which might have been acquired during the process of preparation. The titers given by the polysaccharide and protein were more or less similar after 12 hours' refrigeration. Immediately after incubation, however, the polysaccharide titer was much lower than at the final reading. The protein titers, on the other hand, were much higher after incubation than following refrigeration. No cross reactions occurred between ascaris antigens and antiserums against antigens of *M. expansa* (sheep tapeworm) or vice versa.

Further proof of the difference between the polysaccharide and protein was shown by passive sensitization experiments. Guinea pigs were given intraperitoneally 3 cc. of the antipolysaccharide serum. This was followed eight hours later by intravenous injections of 0.5 cc. of either a 5% solution

of polysaccharide, saline whole worm extract (previously described) or protein. The first two produced typical anaphylactic shock, whereas the protein gave no reaction.

Absorption experiment.—In order to test further the individuality of the protein and polysaccharide fractions, a series of precipitin tests were made on the three types of antiserum (whole worm, protein and polysaccharide) after absorption with either protein or polysaccharide.

Technic.—A preliminary titration was made to determine the approximate optimum concentration and amount of antigen necessary for the removal of homologous precipitins. The following proportions were selected:

- (1) Whole worm antiserum
 - a. Protein, one volume, 1:80
 - b. Polysaccharide, two volumes, 1:100
- (2) Protein antiserum
 - a. Protein, one volume, 1:150
 - b. Polysaccharide (no precipitate) three volumes, 1:200
- (3) Polysaccharide antiserum
 - a. Protein (no precipitate) one volume, 1:150
 - b. Polysaccharide, three volumes, 1:200

These proportions were then added to 10 cc. of the respective serums, incubated at 40 C. for one hour and allowed to stand overnight in the refrigerator. The serums were centrifuged on the following morning and precipitin tests made with the supernatant solutions. Nonspecific reactions were controlled by treating the above antisera with the same proportions of polysaccharide and protein from *M. expansa* and testing with specific ascaris antigens.

Results.—The titers of the various serums before and after absorption are listed in table 2. The first series of tests was made on whole worm anti-

TABLE 2.—*Precipitin Titers with Antisera Against Three Types of Ascaris Lumbricoides Antigens (Whole Worm, Protein and Polysaccharide) Before and After Absorption with the Protein and Polysaccharide Fractions*

Series	Antisera	Treated With;	Highest Active Dilution of Antigen Before and After Absorption of Antisera*					
			Saline extract		Protein		Polysaccharide	
			Before	After	Before	After	Before	After
1	Whole worm	Protein	14,000**	8,500	6,400	0	12,800	12,800
2	Whole worm	Polysaccharide	14,000	10,000	6,400	6,400	12,800	0
3	Polysaccharide	Protein	8,000	5,500	0	0	6,400	3,200
4	Polysaccharide	Polysaccharide	8,000	0	0	0	6,400	0
5	Protein	Protein	16,000	0	25,600	0	0	0
6	Protein	Polysaccharide	16,000	15,500	25,600	25,600	0	0

* Nonspecific controls were negative (see text).

** Numbers represent the highest dilution of antigen showing a reaction after 12 hours at 4 C.

serum absorbed with protein. Before absorption the saline whole worm extract reacted with such serums in dilutions of 1:14,000 and after absorption in dilutions of 1:8,500. The protein-absorbed serum of course gave no reaction with protein. The activity with polysaccharide was not changed. The second series tested the effect of absorbing whole worm antisera with polysaccha-

ride. Reaction with saline whole worm extract was reduced from 1:14,000 to 1:10,000, i.e., the reduction was slightly less than that obtained after protein absorption. The titer with respect to the protein fraction was unaltered and the polysaccharide was zero.

The next two series of tests were made on antipolysaccharide serums. Absorption with protein (series 3) reduced the saline whole worm extract titer from 1:8,000 to 1:5,500 and the protein gave no reaction before or after treatment of the serum. Strangely enough, treatment with protein reduced the polysaccharide titer from 1:6,400 to 1:3,200 although no visible precipitation occurred. The antipolysaccharide serum was completely inactivated by absorption with polysaccharide (series 4).

Absorption of protein antiserum with protein (series 5) left the serum inactive. Absorption with polysaccharide (series 6) slightly reduced the saline whole worm extract titer, but had no effect on the protein titer.

Although there was a slight reaction between the protein fraction and polysaccharide antiserum, it was hardly enough to be proof of protein contamination of the polysaccharide. Since the addition of protein to polysaccharide antiserum produced no precipitate, the reaction must be a type of specific inhibition, possibly brought about by traces of polysaccharide in the protein fraction. Under these conditions the protective action of other elements present might prevent precipitation. On the other hand, it seemed quite significant that the absorption of whole worm antiserum with either polysaccharide or protein left the serum active toward the fraction not used for absorption.

In general, the ability of the polysaccharide to react with appropriate antisera was not changed by boiling in neutral solution for 30 minutes or exposure to pepsin at p_H 4.5 for several days. The activity was slightly decreased after boiling 30 minutes in 0.001 n. NaOH and completely destroyed by boiling in 0.01 n. NaOH.

Skin Tests

Particular attention has been paid within recent years to specific skin sensitiveness in relation to ascaris infections. The active principle involved in the reaction has not been determined. It is of interest, however, that Weinberg and Julien¹⁰ and Ransom et al.¹¹ obtained positive reactions with "non-protein" water-soluble substances. The present study is concerned with the delayed type of reaction occurring in sensitized rabbits.

Preparation of animals and solutions.—Tests were made on whole worm sensitive rabbits previously utilized for precipitin antiserum. They were given one additional series of intravenous injections consisting of saline whole worm extract. The animals' backs were carefully shaved five days before testing and the tests made seven days after the last injection. Each animal received four 0.05 cc. intradermal inoculations, namely, physiological saline, whole worm ascaris extract, ascaris polysaccharide and *M. expansa* polysaccharide. The sites of inoculation were at least 10 cm. apart. The whole

10. Hyg. Viande et Lait 7: 225, 1913.

11. J. Agric. Research 28: 577, 1924.

worm extract was prepared by filtering a 0.9% saline extract of fresh worm material through a Seitz filter. This solution contained approximately 1.0% worm material. The polysaccharide solutions were made in 0.5% concentrations and autoclaved.

Results of skin tests.—Since the reactions were of the delayed type, table 3 lists those occurring after 24 hours. Nonspecific reactions developed in one sensitized animal which reacted to polysaccharide of *M. expansa* and in two normal animals which reacted to ascaris saline extract. These nonspecific reactions disappeared in approximately eight hours.

The results, summarized in table 3, show that although the reaction area of the polysaccharide inoculation was smaller than that of the worm extract,

TABLE 3.—*Skin Reactions Obtained by Intradermal Injections of Polysaccharide or Saline Whole Worm Extract of Ascaris Lumbricoides (from Hog) into Ascaris-sensitive Rabbits*

Rabbit	Inoculation	Skin Reactions*	
		After 24 Hours	After 48 Hours
933	Saline extract Polysaccharide	4 cm. area induration and erythema 2 cm. area slight induration and marked erythema	3 cm. area induration, slight erythema 2 cm. area, erythema, slight necrosis
934	Saline Extract Polysaccharide	4 cm. area induration and erythema 2 cm. area slight induration, erythema	3 cm. area induration and erythema 4 cm. area dark red, slight swelling
935	Saline extract Polysaccharide	5 cm. area slight induration and erythema 2 cm. area slight induration and erythema	2 cm. area induration, no erythema 1 cm. area, dark red, necrosis, no swelling

* Controls for nonspecific reactions were considered negative (see text).

it appeared to be more intense. None of the worm extract inoculations produced necrosis before 48 hours, whereas two out of three polysaccharide inoculations showed small necrotic areas. The polysaccharide gave specific reactions in the three immune animals but none in two normal controls.

Anaphylactic Tests

The first real evidence of a cross reaction between the polysaccharide and protein fractions was brought out by anaphylactic tests. Guinea pigs sensitized with protein were subsequently shocked by intravenous injections of either protein or polysaccharide and those sensitized with polysaccharide were shocked with either protein or polysaccharide. Two explanations are possible; either the polysaccharide contained a trace of antigenic protein or the protein contained enough polysaccharide to produce polysaccharide sensitization. An elucidation of this problem was attempted by studying the quantitative relationships between the two fractions and homologous and heterologous anaphylactins. If animals sensitized with polysaccharide were more sensitive to the polysaccharide than protein, one could validly assume that the cross reaction with protein was due to the polysaccharide (approximately 1%) in the latter fraction. If, however, an amount of polysaccharide were found which sensitized the animals to protein, but not to polysaccharide, it might be assumed that the activity of the polysaccharide was due to protein.

Technic. Two hundred and fifty to 300 gram guinea pigs were given intraperitoneal injections of varying amounts of antigen (see table 4). Twenty-one days later 0.5 cc. of test antigen solution (concentrations as shown in table 4) were administered intravenously into a superficial vein on the posterior surface of the hind leg.

TABLE 4.—*Anaphylactic Tests on Guinea Pigs Sensitized with Either Protein or Polysaccharide from Ascaris Lumbricoides (of Hog): First Part, a Quantitative Study of the Cross Reaction between the Polysaccharide and Protein Fractions, Showing the Minimal Homologous and Heterologous Sensitizing Dose. Second Part, the Effect of Dilute Alkali and Acid upon the Antigenicity of the Polysaccharide*

Group	No. of Animals	Sensitized Intraperitoneally With;	Tested Intravenously With ^{1,2}	Result		
				Died	Symptoms but Recovered	Negative
1	2	Polysaccharide 0.001 gm.	Polysaccharide 0.005 gm.	2		
2	3	0.0001 gm.	0.005 gm.			3
3	3	0.0001 gm.	0.05 gm.	3		
4	5	0.00001 gm.	0.05 gm.	1	3	1
5	3	0.001 gm.	Protein 0.0005 gm.	1	2	
6	6	0.0001 gm.	0.005 gm.			6
7	3	0.0001 gm.	0.005 gm. in 10% NSP ³			3
8	4	Protein 0.001 gm.	Polysaccharide 0.005 gm.			4
9	4	0.001 gm.	0.05 gm.	2	1	1
10	3	0.0001 gm.	0.05 gm.			3
11	3	10 ⁻⁸ gm.	Protein 0.005 gm.	1	2	
12	3	Polysaccharide (untreated) 0.0001 gm.	Polysaccharide (0.05 gm.) boiled 30 min. H ₂ O	2	1	
13	3	0.0001 gm.	0.0001 n. NaOH		3	
14	3	0.0001 gm.	0.01 n. NaOH			3
15	3	(0.01 gm.) boiled 30 min. H ₂ O	(untreated) 0.05 gm.	2	1	
16	3	0.0001 n. NaOH	0.05 gm.			3
17	3	0.01 n. NaOH	0.05 gm.			3
18	4	(0.001 gm.) ⁴ exposed 3 days to pH 4.5, 37 C.	(untreated) 0.05 gm.	1	2	1
19	5	Same, but 1% pepsin added	0.05 gm.	3	2	

(1) There were no nonspecific reactions as tested with polysaccharide from *M. expansa*.

(2) All test injections consisted of 0.5 cc. of solution.

(3) NSP = nonspecific polysaccharide (from *M. expansa*).

(4) Minimal sensitizing dose; 0.0001 gm. failed to sensitize.

Since normal guinea pigs gave reactions with ascaris protein when amounts greater than 0.01 gram were injected intravenously, the test dose never exceeded 0.005 gram. Toxic reactions were never observed with the polysaccharide fraction. Symptoms such as ruffled fur, respiratory failure (coughing, sneezing, etc.), defecation, urination, convulsions and, in the more severe reactions, death, were interpreted as anaphylactic shock. In some instances rather severe reactions were followed by recovery within two to eight hours. When only one or two of the milder symptoms appeared, the test was considered negative.

Results.—The first part of table 4 lists the results obtained from a more or less quantitative study of the anaphylactic tests using polysaccharide and protein fractions. Groups 1, 2 and 3 showed that animals receiving an initial

injection of 0.001 gram of polysaccharide gave positive reactions when subsequently tested with 0.005 gram of polysaccharide, but when the sensitizing amount was reduced to 0.0001 gram the test dose had to be increased to 0.05 gram before a positive reaction was obtained. Group 4 gave the approximate minimal sensitizing dose (0.00001 gram) for polysaccharide when tested with polysaccharide. Animals receiving 0.001 gram of polysaccharide (group 5) were subsequently shocked with 0.0005 gram of protein. Group 6, sensitized with 0.0001 gram of polysaccharide, failed to show any reaction with protein although the amount of the test dose was increased tenfold (from 0.0005 to 0.005 gram). The addition of nonspecific polysaccharide (from *M. expansa*) did not enhance the activity of the protein (group 7). Although polysaccharide-sensitized animals reacted with either protein or polysaccharide, they were more sensitive to the latter.

Guinea pigs sensitized with 0.001 gram of protein (groups 8 and 9) gave positive reactions when tested with 0.05 gram of polysaccharide but not with 0.005 gram. When the sensitizing dose of protein was reduced to 0.0001 gram (group 10), the polysaccharide produced no reaction. The minimal sensitizing dose for protein, when tested with protein (group 11), was the remarkably small amount of 10^{-8} gram. It has been demonstrated, therefore, that although the polysaccharide reacted with protein-sensitized animals, 0.001 gram or more of protein had to be used for sensitization.

The second portion of table 4 illustrates the reaction-capacity of the polysaccharide after being treated with alkali, acid and pepsin. Polysaccharide boiled for 30 minutes in distilled water lost none of its ability to sensitize or give positive reactions (groups 12 and 15). A sample boiled for 30 minutes in 0.0001 n. NaOH gave positive test reactions (group 13), but failed to sensitize (group 16). Similar treatment with 0.01 n. NaOH destroyed all antigenicity (groups 14 and 17).

Preliminary experiments indicated that the minimal sensitizing dose for polysaccharide prepared from pepsin digests (see isolation procedures) was much greater than for other preparations. A sample of the more active product was then subjected to a p_H of 4.5 for three days at 37 C. with and without pepsin. It was found that the reduction of activity was due only to the increase of hydrogen ion concentration and not to any action of the pepsin. The minimal sensitizing dose was increased from 0.00001 gram to 0.001 gram (groups 18 and 19). Such treatment had no apparent effect on the "test antigenicity"¹² of the polysaccharide. (Preparations from peptic digests were used for most of the anaphylactic tests and all the precipitin tests, previously described.)

Various control groups (not listed) gave the following general reactions. Animals sensitized with whole worm material gave positive reactions when tested with either polysaccharide or protein and those sensitized with either fraction were subsequently shocked by injections of saline whole worm extract. No cross reactions were observed with analogous fractions from *M. expansa*.

12. This term is used here to designate specific reaction with antibodies, in contradistinction to inducing antibody formation, i.e., functional antigenicity.

DISCUSSION

Although many of the foregoing data indicated that the polysaccharide from *Ascaris lumbricoides* (of hog) was antigenic, the following facts and possibilities must be taken into consideration. In contrast to many of the bacterial polysaccharides ("haptenes" reacting in high dilutions), the ascaris carbohydrate showed a fairly low reaction-capacity (maximum of 1:25,000) which was very similar to saline whole worm extracts. Furthermore, it was capable of inciting the formation of specific antibodies; a reaction which has generally been interpreted as indicating the presence of a protein contaminant. The fact that chemical analysis for nitrogen was negative merely indicates that not more than approximately 0.015% of protein could be present. (This percentage is based upon the assumption that more than 0.0002 gm. of nitrogen would have been detected in the analysis of a 10 gm. sample of polysaccharide.) Thus, 0.00001 gm. of polysaccharide (minimal sensitizing dose for guinea pigs, table 4) might contain but slightly less protein than was found to be the minimum (10^{-8} gm.) required for the sensitization of guinea pigs with the protein fraction. With regard to the production of precipitins, Hektoen and Cole¹³ found that a fairly high titer serum could be produced by approximately 0.0003 gm. of protein, an amount only slightly greater than might have been present in 0.4 gm. of polysaccharide (the amount used for the sensitization of rabbits, table 1). All such factors suggest the possibility of traces of antigenic protein.

Boiling in neutral solution and treatment with proteolytic enzymes, which had no marked effect on the antigenicity of the polysaccharide, cannot be considered adequate to rule out the presence of proteins because both reactions may not only fail to go to completion, but may be partially reversible, and because some soluble antigenic proteins are not heat-coagulable. This also applies to chemical tests for protein since such tests are much less sensitive than immunological reactions. The only evident method, namely that of determining whether or not cross reactions occurred between polysaccharide and protein, had two failings. First, the obvious possibility that a protein contaminant of the polysaccharide might be lacking in the particular protein fraction studied. (This, however, seems very unlikely since the protein fraction used was closely associated with the polysaccharide.) The second difficulty lay in securing a polysaccharide-free protein preparation without causing denaturation. This was not entirely overcome and the preparation studied contained approximately 1% polysaccharide material.

A study of the protein and polysaccharide by precipitin reactions showed that they were immunologically distinct since both reacted only with homologous and whole worm antisera and were capable of absorbing only their homologous antibodies from the whole worm antiserum. On the other hand, anaphylactic tests did not indicate any such sharp distinction. It is believed, however, that the polysaccharide of the protein fraction played an important rôle in the cross reactions. For example, guinea pigs had to receive at least 10^{-3} gram of protein in order to give a positive reaction with poly-

13. J. Infect. Dis. 50: 171, 1932.

saccharide. This amount of protein (10^{-3} gm., of which 1% was polysaccharide) would contain approximately 10^{-5} gram of polysaccharide, which was the minimal carbohydrate sensitizing dose. It is difficult to explain, however, why polysaccharide-sensitized animals gave positive reactions with the protein fraction since the amount of polysaccharide in the protein fraction was less than required for positive reactions. Perhaps, a slight anaphylactic shock, induced by the polysaccharide, plus the toxicity of the protein¹⁴ gave a combined effect interpreted as anaphylactic shock.

The most conclusive evidence of the individuality of the protein and polysaccharide fractions is based upon the data from two groups of guinea pigs (table 4, groups 4 and 6) which received 10^{-5} and 10^{-4} gram of polysaccharide respectively. Since chemical analysis showed that the polysaccharide contained not more than 0.015% protein, 10^{-5} gram of polysaccharide (minimal sensitizing dose when tested with polysaccharide) would contain less protein than was indicated for the minimal sensitizing dose of protein (10^{-8} gm.) when tested with protein. These data are in favor of a polysaccharide-sensitization, but it must be admitted that the difference between the possible protein content of 10^{-5} gram of polysaccharide and the minimal protein sensitizing dose was relatively small. This difference, however, was made much greater by the results from group 6 which received 10^{-4} gram of polysaccharide, but gave negative reactions when subsequently tested with protein. It may be concluded, therefore, that 10^{-4} gram of polysaccharide did not contain the minimal protein-sensitizing dose. Thus, a polysaccharide-sensitization is indicated by the results from groups 4 and 6.

The induction of antibodies by the polysaccharide cannot be taken as proof for the presence of protein since considerable evidence has been presented, within recent years, tending to show that polysaccharides may, under certain conditions, function as complete antigens. For example, Zozaya¹⁵ was able to induce antibody formation by the injection of polysaccharides adsorbed on collodion particles. Protective antibodies have also been produced by Zozaya and Clark¹⁶ and Avery and Goebel¹⁷ by the injection of pneumococcal-polysaccharides. These investigations, along with other less convincing reports, indicate that protein may not be essential for sensitization.

One other interesting finding brought out by the present investigation was the effect of dilute acid and alkali on the polysaccharide. The ability to incite the formation of antibodies was lost after boiling in 0.0001 n. NaOH and reduced by three day exposure to p_H 4.5 at 37 C. No effect was noted, however, on the capacity of the polysaccharide to give specific test reactions. For some unknown reason the ability to incite the formation of antibodies was lost more easily than the capacity to react with them. In some respects this reaction resembles that of S.S.S. type I pneumococcus, which was found

14. The amount of protein used for anaphylaxis was very close to the toxic dose for guinea pigs. (See protein isolation.)

15. J. Exper. Med. **55**: 325, 1932.

16. J. Exper. Med. **56**: 21, 1933.

17. J. Exper. Med. **58**: 781, 1933.

by Avery and Goebel¹⁷ to be deacetylated by the action of alkali. When the acetyl group was present, protective antibodies were induced and when absent, the polysaccharide lost this power and became a "haptene."

In general, the foregoing results offer three interpretations as to the immunological activity of the polysaccharide fraction. (1) The polysaccharide was itself a complete antigen. (2) Its ability to sensitize animals was due to the presence of minute amounts of protein, but it reacted as a "haptene" with specific antibodies. (3) All the reactions were accounted for by traces of protein. Of the three possible explanations, the latter is the least evident.

There is no doubt but that the polysaccharide fraction described herein from *Ascaris lumbricoides* (of hog) was antigenic. Furthermore, most of the data tend to show that the activity was due to the polysaccharide itself and not some contaminant. Possible contamination with minute traces of antigenic proteins must, however, be considered since the complete absence of such substances cannot be ascertained until chemical methods become as sensitive as biological reactions.

SUMMARY

A polysaccharide from *Ascaris lumbricoides* (of hog) has been isolated and purified. Its general chemical characteristics and composition may be summarized as follows:

(a) *Before acid hydrolysis*.—It is very soluble in hot or cold water and forms a neutral opalescent solution which gives a red-brown iodine reaction but no color tests for protein. It is precipitated by barium and calcium hydroxides, but not by mineral acids, sodium hydroxide or basic lead acetate. It is highly dextrorotatory, does not reduce Fehling's solution, contains no chemically detectable nitrogen and only a trace of ash.

(b) *After acid hydrolysis*.—The hydrolysate shows a maximum reducing power of 94%, calculated as glucose and has a specific rotation of approximately $+57^\circ$. Color reactions for hexuronic acids, pentoses and ketoses are negative. The hydrolysate is tentatively considered to consist entirely of glucose.

Experiments on precipitin, skin and anaphylactic tests indicate that the polysaccharide is capable of inducing the formation of specific antibodies as well as reacting with them to give specific test reactions. The reactions are immunologically specific with respect to a similar polysaccharide from *M. expansa* (sheep tapeworm).

The immunological properties of the polysaccharide are not affected by boiling in neutral solutions or by proteolytic enzymes. The ability of the polysaccharide to incite specific antibody formation, i.e., functional antigenicity, is more easily destroyed by the action of dilute acids and alkali than is the capacity to react with them, i.e., test antigenicity.

The possibility that the immunological activity of the polysaccharide might be due to minute traces of protein is considered. The immunological relationship found between the polysaccharide and the closely associated water-soluble protein fraction is described and may be summarized as follows:

(a) *Precipitin tests*.—Anti-whole-worm serum precipitates both the polysaccharide and protein.

Absorption of such antisera with protein removes the precipitins for protein but not for polysaccharide.

Absorption of such antisera with polysaccharide removes the precipitins for polysaccharide but not for protein.

The addition of protein to anti-polysaccharide serum produces no precipitate, but decreases the activity toward polysaccharide.

Anti-polysaccharide sera react with the polysaccharide but not with protein, and anti-protein serum reacts with protein but not with polysaccharide.

(b) Anaphylactic tests.—Guinea pigs sensitized with polysaccharide are more sensitive to polysaccharide than to protein. The minimal sensitizing dose is 0.001 gm. when tested with protein, but only 0.00001 gm. when tested with polysaccharide.

Guinea pigs sensitized with protein are more sensitive to protein than to polysaccharide. The minimal sensitizing dose is 0.001 gm. when tested with polysaccharide, but only 10^{-8} gm. when tested with protein.

Cross reactions between the two fractions can, for the most part, be accounted for by the presence of a detectable amount of polysaccharide in the protein fraction.

The results obtained so far not only indicate the immunological significance of polysaccharides from animal parasites, but also suggest a source from which large quantities of polysaccharide can be obtained for immunochemical study.

